SHORT COMMUNICATION

SECONDARY METABOLITE FROM ENDOPHYTIC FUNGI *Aspergillus niger* OF THE STEM BARK OF KANDIS GAJAH (*Garcinia griffithii*)

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ABSTRACT

*Garcinia griffithii are known as kandis gajah including the Garcinia genus. This plant has been traditionally used by local communities Sarasah Bonta, Lembah Arau, West Sumatra, to treat various diseases including gout. Aspergillus niger was isolated from the tissues of the stem bark of Garcinia griffithii. The fungi strain was identified base on colony and cell morphology characteristic. Aspergillus niger cultured in media 5 L Potatos Dextose Broth (PDB) for 8 weeks and filtered. Media that already contains secondary metabolites are partitioned using ethyl acetate solvent in 5 L (twice), followed by evaporation. Furthermore, the extract is separated by chromatographic techniques to obtain a pure compound form of white crystal. The molecular structure of compound isolation result are determined by spectroscopic methods including IR, ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC, and COSY. The compound was determined as phenolic (**1**).*

Keywords: *endophytic fungi; Aspergillus niger; Garcinia griffithii*

ABSTRAK

*Tumbuhan Garcinia griffithii yang dikenal dengan nama kandis gajah termasuk ke dalam genus Garcinia. Tumbuhan ini telah digunakan secara tradisional oleh masyarakat setempat yaitu di daerah Sarasah Bonta, Lembah Arau, Sumatera Barat, untuk mengobati berbagai penyakit termasuk asam urat. Jamur endofitik Aspergillus niger telah diisolasi dari jaringan kulit batang Garcinia griffithii. Strain jamur ini diidentifikasi berdasarkan karakteristik koloni dan morfologi sel. Aspergillus niger selanjutnya dikultur dalam 5 L media Potatos Dextose Broth (PDB) selama 8 minggu dan disaring. Media yang telah mengandung metabolit sekunder dipartisi menggunakan 5 L pelarut etil asetat (dua kali ulangan) yang dilanjutkan dengan evaporasi. Selanjutnya, ekstrak ini dipisahkan dengan teknik kromatografi untuk mendapatkan senyawa murni berupa kristal putih. Struktur molekul senyawa murni ditentukan dengan metode spektroskopi meliputi IR, ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC, dan COSY. Senyawa hasil isolasi ditentukan sebagai senyawa turunan fenolat (**1**).*

Kata Kunci: *jamur endofitik; Aspergillus niger; Garcinia griffithii*

INTRODUCTION

Plant endophytic fungi are defined as the fungi which spend the whole or part of their lifecycle colonizing inter-and/or intra-cellularly inside the healthy tissues of the host plants, typically causing no apparent symptoms of disease. They are important components of plant micro-ecosystems [1-3]. Plant endophytic fungi have been found in each plant species examined, and it is estimated that there are over one million fungal endophytes existed in the nature. Plant endophytic fungi have been recognized as an important and novel resource of natural bioactive products with potential application in agriculture, medicine and food industry [4-6]. Some endophytes have the ability to produce the same or similar bioactive compounds as those originated from their host plants [7].

Garcinia is the most important genus of Guttiferae family, widely distributed in tropical Africa, Asia, New Caledonia and Polynesia. The genus *Garcinia* is well known to be rich in variety of oxygenated and prenylated phenol derivatives [8]. Prenylated xanthones and their structurally related benzophenones often exhibit a wide range of biological and pharmacological activities, e.g., antioxidant, cytotoxic, anti-inflammatory, antifungal, and inhibitory

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effects on xanthine oxidase and monoamine oxidase [9]. As part of a phytochemical study of our research, we have previously reported four xanthones, such as 1,5-dihydroxy-3,6-dimethoxy-2,7-diprenylxanthone which is active as an antimalarial compound; 1,7-dihydroxyxanthone; 1,6-dihydroxy-3-methoxy-4,7-diprenylxanthone; 1,6,7-trihydroxyxanthone, and two benzophenones isoxanthochymol and guttiferone I which is active as antioxidant compounds. Other than that has been isolated two steroid glucoside β-sitosterol-3-O-β-D-glucoside and stigmasterol-3-O-β-D-glucoside [10-14].

The purpose of the present study was to isolation and determines of endophytic fungi from stem bark of *G. griffithii* growing wild in Lembah Arau, West Sumatra, continued extract, explore and structure elucidation natural product produced by the endophytic fungi *Aspergillus niger.*

EXPERIMENTAL SECTION

Materials

Medium for isolation endophytic fungi: nutrient agar (NA), nutrient broth (NB), potato dextrose broth (PDB), potato dextrose agar (PDA), a series medium for physiologies assay or fungi identification, column chromatography using Si gel 60 (70-230 mesh.). Analytical thin layer chromatography (TLC) was carried out using Merck (Art.5554) silica gel 60 F_{254}, pre-coated aluminium sheets solvents for chromatography were technical grade and distilled before use.

Plant material

The stem barks of *G. griffithii* were collected on April 2010 from the Sarasah Bonta, Lembah Arau, Kabupaten Lima Puluh Kota, West Sumatra. This plant was identified and voucher specimen has been deposited in the Laboratory of Herbarium Universitas Andalas (ANDA), Padang.

Instrumentation

The apparatus in the research were counter colony, autoclave, incubator, water bath, refrigerator, microscope, magnetic hotplate, UV lamp, column chromatography and generally apparatus in organic and microbiology laboratory. Melting point was determined on a micromelting point apparatus, IR spectra were recorded with FTIR-8400 Shimadzu, NMR spectra were recorded at 500 MHz (^{1}H) and 125 MHz (^{13}C) on JEOL JNM ECA-500 spectrometer. Organic materials were detected by first viewing the plate under UV light at 254 nm and 365 nm. The extracts were organic mixture samples were applied in the pre-adsorbed form on silica gel 60 (70-230 mesh).

Procedure

Fungi isolation

Isolation of endophytic fungi was standardized and modified based on the method described by Debbab et al. [15]. Stem barks were rinsed in sterilized distilled water twice. Surface sterilization was done by immersing the stems in 70% ethanol for 2 min (twice) followed by rinsing again twice in sterilized distilled water. Then, the stem barks were cleaved aseptically into small segments (≈ 1 cm in length). The material was placed on a Petri dish containing potato dextrose agar medium (PDA) supplemented with 100 µg/mL of chloramphenicol to suppress bacterial growth and incubated at room temperature (25 °C). After several days hyphae growing from the plant material were transferred to other plates, incubated again for 10 days, and periodically checked for culture purity.

Characterization of endophytic fungi

The fungi strain was identified base on colony and cell morphology characteristic [16]. Characterization of macroscopic based on colony morphology by growing the isolates of the fungus in the three medium is different and medium CDA (*Czapez Dox Agar*), MEA (*Malt Extract Agar*), and PDA (*Potato Dextrose Agar*). Characterization of microscopic based on cell morphology by observing the non-reproductive structures, reproductive structures, conidia, and conidiophores.

Potato dextrose broth (PDB) culture of isolated fungi

Ten flasks (1 L each) containing 500 mL of PDB medium were autoclaved. A small part of the medium from a petri dish containing the purified fungi was transferred under sterile conditions to the PDB medium. The fungi strain was grown on PDB medium at room temperature for two months.

Extraction, exploration, and structure elucidation

The culture was extracted with 5 L ethyl acetate (twice). Evaporation of the extract gave a dry extract which was chromatographed over a silica gel column with n-hexane : ethyl acetate as solvent (gradient elution). Based on detection by TLC (SiO gel F254, Merck, Darmstadt, Germany) using n-hexane:EtOAc (5:5) as a solvent system, collected fractions were combined, and subjected to recolumn over a silica gel with n-hexane : ethyl acetate (5:5) to obtain pure compound. Identification of the molecular structure is done by spectroscopic methods including IR, ^{1}H-NMR,

Tabel 1. The NMR data of compound 1, recorded at ^{1}H-500 MHz; ^{13}C-125 MHz in methanol-d_4

No. C	δ_C ppm	δ_H ppm (ΣH, multiplicity, *J* Hz)	HMBC	COSY
1	130.3	7.22 (1H;m)	128.0	
2	129.7	7.27 (1H;m)	130.3; 138.2	
3	129.7	7.27 (1H;m)	130.3; 138.2	
4	128.0	7.20 (1H;m)	39.4	
4a	166.9			
6	176.6			
7	30.2	2.56 (2H;s)	176.6	
7a	49.3			
8	173.0			
9	173.4			
10	89.1	5.56 (1H;d;2.0)	101.9; 173.4	6.00
11	101.9	6.00 (1H;d;2.0)	89.1; 164.4; 173.4	5.56
12	164.4			
12a	54.2	4.90 (1H;m)	39.4; 49.3; 164.4; 173.0	2.97; 3.16
13	39.4	13A: 2.97 (1H;dd;13.6;5.2)	130.3; 138.2; 164.4; 54.2	3.16; 4.90
		13B: 3.16 (1H;dd;13.6;7.1)	130.3; 138.2; 164.4; 54.2	2.97; 4.90
13a	138.2			
9-OCH_3	57.1	3.82 (3H;s)	173.4	
7a-CH_3	22.4	1.91 (3H;s)	173.0	

Fig 1. Morphological characterization of isolates of the fungi cells (a = conidia; b = conidiophores)

^{13}C-NMR, HMQC, HMBC, and COSY (NMR spectra were recorded at 500 MHz (^{1}H) and 125 MHz (^{13}C) on JEOL JNM ECA-500 spectrometer).

RESULT AND DISCUSSION

Base on its characterization, the isolate is *Aspergillus niger*. This result is consistent with reported by Khan et al. [17]. *Aspergillus niger* grew rapidly on CDA, MEA, and PDA, with the diameter 4-5 cm in 7 days. Colonies were black in color. Conidiophores were smooth with thick walls, unseptate. They were white to yellowish colour near the vesicle. Conidial heads were fuscous black, globose, up to 3 µm in diameter. Vesicles were colorless and globose, thick-walled. Conidial chains were present over the entire surface of vesicles. Conidia were rough, globose and 3 µm in diameter. The results of morphological characterization of isolates of the fungus cells are shown in Fig. 1.

Ethyl acetate extract which containing secondary metabolite was concentrated under reduced pressure to give crude extract 7.2 g. TLC results of EtOAc extract that indicates that there is one major component. Column chromatography of EtOAc extract on silica gel with n-hexane-EtOAc of increasing polarity as eluent. Fraction which gave the same Rf on TLC were combined and gave five column fractions (F1-F5). F3 fraction was further rechromatographed with n-hexane-EtOAc (8:2 – 1:9) to afford 48 fractions which were combined to four fractions (F3.1-F3.4). F3.3 fraction purified by rechromatographed to yield compound **1** (102 mg).

Compound **1** was isolated as a white crystal, m.p 202 – 204 °C. IR υ_{max} (KBR) cm^{-1} : 3294.2 cm^{-1} and 1033.8 cm^{-1} (O-H), 3087.8-3003.0 cm^{-1} (Ar-H), 2950.9-2850.6 cm^{-1} (C-H), 1735.8 cm^{-1} (C=O), 1651.0, 1566.1, 1544.9 cm^{-1} (C=C Ar), 1159.1 cm^{-1} (C-O ester). The ^{1}H-NMR spectra of **1** revealed 12 signals: δ_H 1.91 (3H;s); 2.56 (2H;s); 2.97 (1H;dd;13.6;5.2); 3.16 (1H;dd;13.6;7.1); 3.82 (3H;s); 4.90 (1H;m); 5.56 (1H;d;2.0); 6.00 (1H;d;2.0); 7.20 (1H;m); 7.22 (1H;m); 7.27 (1H;m); 7.27 (1H;m). The ^{13}C-NMR spectra of **1** revealed 18 signals: seven sp^3 carbon (at δ_C 101.9; 128.0; 129.7; 129.7; 130.3; 138.2; 164.4; 166.9; 173.0; 173.4; 176.6 ppm). Complete assignment of carbon and proton were made by analysis 2D NMR (Table 1).

The position of the proton were assigned from HMQC and HMBC spectrum (Table 1), methyne proton sp^3 at δ_H 4,90 ppm (1H;m) in spectrum HMBC showed four correlation [^{2}J: 39.4 (C-13); 164.4 (C-12); 49.3 (C-7a); ^{3}J: 173.4 (C-8), which placement at C-12a. The vinyl proton at δ_H 7.20 (1H;m), at C-4 position and at HMBC spectrum showed correlation ^{4}J: 39.4 (C-13). The other one vinyl proton at δ_H 6.00 (1H, *d, J* = 2.0) be

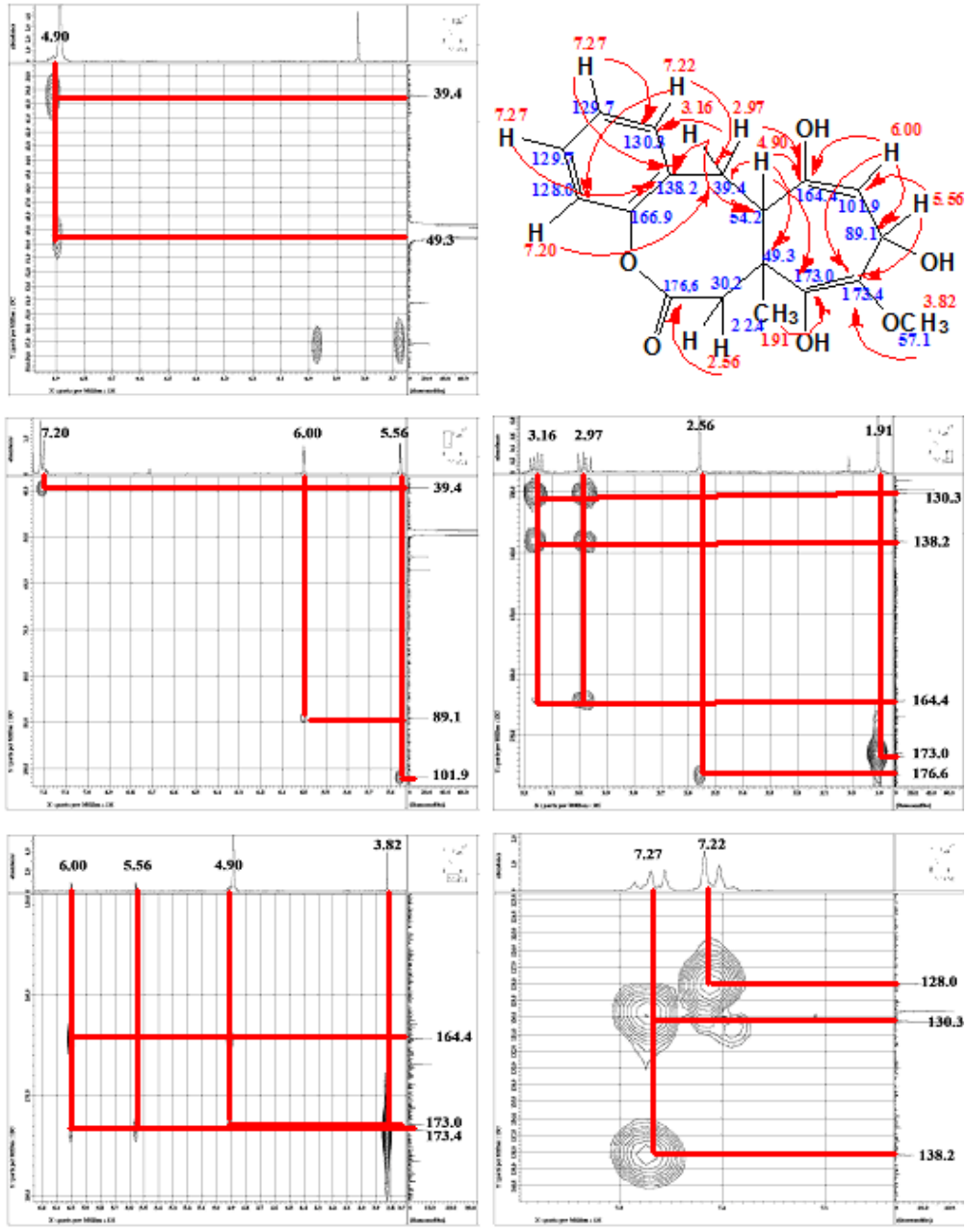

Fig 2. The HMBC correlation and δ-assignment of compound **1**

coupled with one methine proton at δ_H 5.56 (1H, *d*, *J* = 2.0) in HMBC spectrum showed correlation with carbon [^2J: 164.4 (C-12); 89.1 (C-10)]; [^3J: 173.4 (C-9)], and the proton at δ_H 5.56 ppm [^2J: 101.9 (C-11); 173.4 (C-9)]. The methylene proton at δ_H 2.97 (1H;dd;13.6;5.2) and 3.16 (1H;dd;13.6;7.1) showed correlation with carbon [^2J: 138.2 (C-13a); 54.2 (C-12a)]; [^3J: 164.4 (C-12); 130.3 (C-1)] which indicated the placed of C-13A and C-13B. Two signals singlet of two proton each at δ_H 1.91 (3H;s) and δ_H 3.82 (3H;s) showed correlation [^3J: 173.0 (C-8)] and [^3J: 173.4 (C-9) respectively be placed at 49.3 (7a-CH_3) and 173.4 (9-OCH_3). This suggests that the two protons are methyl and methoxyl groups. The presence of four aromatic protons indicated by two signal at δ_H 7.27 (1H;m), and the other at 7.22 (1H;m) and 7.20 (1H;m). Existence of two aromatic protons attached to two carbon has the same chemical shift at 129.7 ppm indicates that the position of each proton is symmetrically. Whereas the methylene proton sp^3 at δ_H 2.56 (2H;s) at HMBC spectrum showed correlation with carbon at [^2J 176.6 (C-6)]. The HMBC correlation and δ-assignment of compound **1** showed Fig. 2.

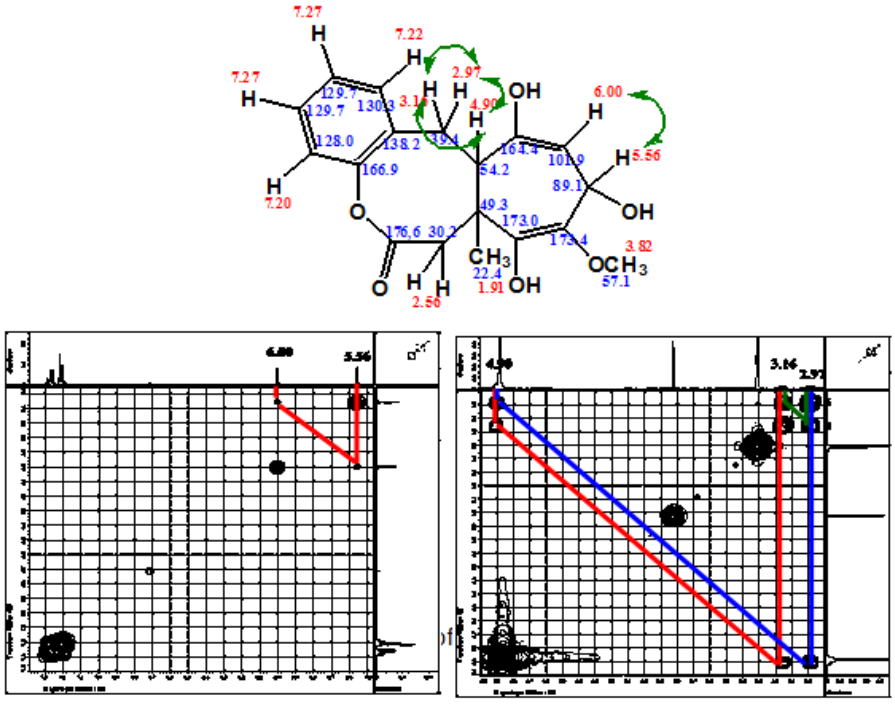

Fig 3. The COSY correlation of compound **1**

Furthermore, the ^{1}H-^{1}H COSY spectrum (Fig. 3) showed there was correlations proton with proton in compound **1**. The vinyl protons that at δ_H 6.00 ppm (H-11) showed correlation with methyne proton at δ_H 5.56 ppm (H-10). The sp^3 methyne protons that at δ_H 4.90 ppm (H-12a) showed correlation with methylene proton at δ_H 2.97 (H-13A) and 3.16 ppm (13B).

By combining the results obtained by the spectrometric methods (IR, ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC and COSY) was proposed $C_{18}H_{20}O_6$ as the molecular formula for compound **1**, The molecular formula indicated nine degrees of unsaturation. The NMR spectrums for **1** revealed that seven of the nine unsaturation degrees were attributed to one carbonyl, and five double bond. Thus, the remaining unsaturation degree came from a ring. The compound **1** as 8,10,12-trihydroxy-9-methoxy-7a-methyl-7,7a,12a,13-tetrahydrobenzocycloheptaoxocin-6-one.

CONCLUSION

The endophytic fungi *Aspergillus niger* from the stem bark of kandis gajah (*Garcinia griffithii*) has produced a secondary metabolite. Based on NMR spectroscopic analysis including IR, ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC and COSY, the compounds is 8,10,12-trihydroxy-9-methoxy-7a-methyl-7,7a,12a,13-tetrahydrobenzocycloheptaoxocin-6-one.

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